\TENT COOPERATION TRE ˙Y

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing: 10 June 1999 (10.06.99)	
International application No.: PCT/US98/16379	Applicant's or agent's file reference: 98,506-A
International filing date: 06 August 1998 (06.08.98)	Priority date: 06 August 1997 (06.08.97)
Applicant: FRACE, A., Michael et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

01 March 1999 (01.03.99)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as designated Office

Date of mailing:
28 April 1999 (28.04.99)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/US98/16379	

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

C[illegible]r the designated Office (DO/US)

TF PATE[illegible] COOPERATION TREA[illegible]

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION IN CASES FOR WHICH NO OTHER FORM IS APPLICABLE

To:

GREENFIELD, Michael, S.
McDonnell Boehnen Hulbert & Berghoff
Suite 3200
300 South Wacker Drive
Chicago, IL 60606
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing (*day/month/year*) 28 April 1999 (28.04.1999)	
Applicant's or agent's file reference 98,506-A	**REPLY DUE** see paragraph 1 below
International application No. PCT/US98/16379	International filing date (*day/month/year*) 06 August 1998 (06.08.1998)
Applicant CENTERS FOR DISEASE CONTROL AND PREVENTION	

1. ☐ REPLY DUE within _____ months/days from the above date of mailing

☐ NO REPLY DUE, however, see below

☒ IMPORTANT COMMUNICATION

☐ INFORMATION ONLY

2. COMMUNICATION:

The applicant, in respect of the above-identified international application, is notified that the receiving Office (RO/US) has informed the International Bureau that an erroneous international filing date was stamped on the first page of the Request Form PCT/RO/101.

Please correct all notifications previously sent by the International Bureau to indicate the correct international filing date of **06 August 1999 (06.08.98)**, instead of 03 September 1998 (03.09.98).

Please also note that due to the change of the international filing date, the above identified international application has not been published promptly after the expiration of 18 months from the priority date, as provided in PCT Article 21(2)(a).

The international publication will take place on 10 June 1999 (10.06.99).

Meanwhile, the International Bureau will communicate a copy of the international application to the designated Offices concerned, in accordance with PCT Article 20.

A copy of this notification is being sent to the receiving Office (RO/US) and the designated Offices concerned.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Ting Zhao
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

TENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
98,506-A		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 98/ 16379	03/09/1998	06/08/1997
Applicant		
CENTERS FOR DISEASE CONTROL AND PREVENTION et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing
   - [X] filed with the international application.
   - [ ] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [X] the text is approved as submitted by the applicant
   - [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____
   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figur better characterizes the invention.
   - [X] None of the figures.

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 98/ 16379

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 52-56 and 62-66 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/16379

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/44 C07K14/08 C07K19/00 C12N15/62 C12N15/70
C12N1/21 A61K39/145 C07K16/10 A61K39/42 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRAMBAS S ET AL: "Influence of amantadine resistance mutations on the pH regulatory function of the M2 protein of influenza A viruses." VIROLOGY, (1992 DEC) 191 (2) 541-9. JOURNAL CODE: XEA. ISSN: 0042-6822., XP002085466 United States see abstract see page 541, right-hand column, paragraph 3 - page 542, left-hand column, paragraph 2 see page 543, left-hand column, paragraph 3 - page 545, left-hand column, paragraph 1 see page 548, left-hand column, paragraph 3 - page 549, left-hand column, paragraph 2 --- -/--	9,16,21, 25,30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 November 1998	08/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HOLSINGER L J ET AL: "Influenza A virus M2 ion channel protein: a structure-function analysis." JOURNAL OF VIROLOGY, (1994 MAR) 68 (3) 1551-63. JOURNAL CODE: KCV. ISSN: 0022-538X., XP002085467 United States see abstract see page 1553, left-hand column, last paragraph - right-hand column, paragraph 3 see page 1557, right-hand column, paragraph 2 - page 1559, right-hand column, paragraph 1 see page 1559, right-hand column, paragraph 3 - page 1562, left-hand column, paragraph 1 ---	9,16,21, 25,30
X	WANG C ET AL: "Activation of the M2 ion channel of influenza virus: a role for the transmembrane domain histidine residue." BIOPHYSICAL JOURNAL, (1995 OCT) 69 (4) 1363-71. JOURNAL CODE: A5S. ISSN: 0006-3495., XP002085468 United States see abstract see page 1364, left-hand column, paragraph 2 ---	9,16,21, 25,30
A	WO 93 03173 A (THE UNITED STATES OF AMERICA) 18 February 1993 see the whole document ---	1-66
A	WANG C ET AL: "Direct measurement of the influenza A virus M2 protein ion channel activity in mammalian cells." VIROLOGY, (1994 NOV 15) 205 (1) 133-40. JOURNAL CODE: XEA. ISSN: 0042-6822., XP002085469 United States see abstract see page 134, left-hand column, paragraph 1 see page 138, right-hand column, paragraph 2 - page 139, right-hand column, paragraph 2 -----	1-66

INTERNATIONAL SEARCH REPORT

[redacted]ion on patent family members

[redacted]nal Application No
[redacted]T/US 98/16379

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9303173 A	18-02-1993	US 5290686 A	01-03-1994
		AU 659867 B	01-06-1995
		AU 2405692 A	02-03-1993
		CA 2111116 A	18-02-1993
		EP 0597008 A	18-05-1994
		JP 6509710 T	02-11-1994

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
McDONNELL BOEHNEN HULBERT
& BERGHOFF
Attn. GREENFIELD, M.
300 South Wacker Drive, Suite 3200
CHICAGO, IL 60606
UNITED STATES OF AMERICA

Date of mailing *(day/month/year)*	08/12/1998
Applicant's or agent's file reference: 98,506-A	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/US 98/ 16379	International filing date *(day/month/year)*: 03/09/1998

Applicant: CENTERS FOR DISEASE CONTROL AND PREVENTION et al.

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicants's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Andria Overbeeke-Siepkes

Form PCT/ISA/220 (January 1994)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

GREENFIELD, M.
McDONNELL BOEHNEN HULBERT
& BERGHOFF
300 South Wacker Drive, Suite 3200
CHICAGO, IL 60606
ETATS-UNIS D'AMERIQUE

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL PRELIMINARY
EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	15. 11. 99
Applicant's or agent's file reference 98,506-A	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/16379	06/08/1998	06/08/1997

Applicant
CENTERS FOR DISEASE CONTROL AND PREVENTION et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

19

PATENT COOPERATION TREATY

REC'D 18 NOV 1999
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
98,506-A		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US98/16379	06/08/1998	06/08/1997
International Patent Classification (IPC) or national classification and IPC C12N15/44		
Applicant CENTERS FOR DISEASE CONTROL AND PREVENTION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
01/03/1999	15. 11. 99
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Herrmann, K Telephone No. +49 89 2399 2670

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US98/16379

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-28 as originally filed

Claims, No.:

1-23 with telefax of 22/09/1999

Drawings, sheets:

1/4-4/4 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☒ the claims, Nos.: 24-66
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

see separate sheet

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has

been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:

see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 21, 23.

because:

☒ the said international application, or the said claims Nos. 21, 23 relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inv ntive st p or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-8, 10-12
	No:	Claims 9, 13-20, 22
Inventive step (IS)	Yes:	Claims 1-8, 10-11
	No:	Claims 9, 12-20, 22
Industrial applicability (IA)	Yes:	Claims 1-20, 22
	No:	Claims

2. Citations and explanations

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

Citations

The documents mentioned in this International Preliminary Examination Report (IPER) are numbered as in the International Search Report dated 24.11.98, i.e. **D1** and **D5** correspond to the first and the last document of the search report, respectively.

Re ITEM I (Basis of the opinion)

1 The application contains a sequence listing consisting of 2 pages (5 sequences).

2 The amended claims 1-23 filed with telefax of 22.09.99 can be regarded as meeting the requirements of Art. 19(2) PCT.

Re ITEM II (Priority)

The priority document pertaining to the present application was not available at the time this IPER was established. Hence, the current assessment is based on the assumption that all claims enjoy priority rights from the filing date of the priority document (06.08.97).

Re ITEM III (Non-establishment of opinion)

As far as the subject-matter of claims 21 and 23 is directed to *in vivo* treatment or diagnostic methods on the human or animal body, it is excluded from examination by Art. 34(4)(a)(i) PCT in combination with Rule 67.1(iv) PCT.

No unified criteria exist among the PCT member states for the assessment whether the treatment of the human or animal body is industrially applicable or not. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but will allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re ITEM V (Novelty, inventive step, industrial applicability)

1 **Novelty (Art. 33(2) PCT)**

1.1 The subject-matter of claims 1-8 and 10-12 has not been made available to the public by any of the available prior art documents and can therefore be regarded as novel.

1.2 The subject-matter of claims 9, 13-20 and 22 does not meet the requirements of Art. 33(2) and 33(3) PCT.

Claim 9 requires that at least "one" amino acid residue of the transmembrane region is replaced with neutral or hydrophobic amino acid residues. The replacement of amino acid residues adjacent to the transmembrane region on the C-terminal side is optional ("0 to 12"). The IPEA is of the opinion that the terms "reduced" and "enhanced" are relative and are thus not regarded as technical features which are sufficient to establish novelty of the polypeptide *per se*.

1.3 **D1** discloses a modified M2 polypeptide comprising a sequence of amino acids identical to a native M2 protein in which e.g. Isoleucin at position 27 has been repalced with the hydrophilic amino acid residue serine (see D1, Table 1). The modified M2 polypeptides disclosed in **D1** are therefore novelty-destroying for the subject-matter of claim 9. Similar results have been disclosed in **D2** (Fig. 1) and **D3** (p. 1364, left col., 2nd par.: replacement of histidine with alanine, glutamate or glycine) which therefore also deprive said claim of novelty.

1.4 In view of the novelty-objection raised against independent claim 9, the further embodiments characterized in independent claims 13-16, 19 and 22 and dependent claims 17 and 18 are also not novel over the subject-matter disclosed in **D1**, **D2** or **D3**.

1.5 **D1** discloses an antiserum against an M2 polypeptide (see D1, p. 543, 1st par.: "against a C-terminal peptide of M2"). It is self evident that an antiserum against a certain polypeptide contains an antibody against that polypeptide. There is no doubt to the IPEA that the antiserum/antibody of **D1** recognizes the "modified M2

polypeptide" defined in present claims 1-12. Therefore, the subject-matter of claims 20 cannot be regarded as meeting the requirements of Art. 33(2) and (3) PCT.

2 **Inventive step (Art. 33(3) PCT)**

2.1 The subject-matter of claims 1-8 and 10-12 cannot be derived from the available prior art in an obvious manner and therefore complies with the requirements of Art. 33(3) PCT.

2.2 The subject-matter of claim 12 (*native* M2 protein from the A/Aichi/2/68 (H3N2) virus) and consequently the subject-matter of claims 14-19 and 22 does not contribute to an inventive solution of an unexpected technical problem. Said claims contain subject-matter which is considered merely an obvious modification to a person skilled in the art.

3 **Industrial applicability (Art. 33(4) PCT)**

Claims 1-20 and 22 meet the criteria as set forth by Art. 33(4) PCT.

Re ITEM VII (Certain defects in the international application)

Independent claims 1, 7 and 9 are all directed to "a modified M2 polypeptide" (Art. 6 PCT in combination with Rule 6.4 PCT).

We claim:

1. A modified M2 polypeptide with reduced hydrophobicity and enhanced recombinant expression relative to a native M2, the modified M2 polypeptide comprising a sequence of amino acids identical to a native M2 protein in which the transmembrane region and from 0 to 12 amino acid residues adjacent to the transmembrane region on the C-terminal side have been deleted.

2. The modified M2 polypeptide of claim 1, wherein the transmembrane region and none of the adjacent residues on the C-terminus side of the transmembrane region have been deleted.

3. The modified M2 polypeptide of claim 1, wherein the transmembrane region and the adjacent 12 amino acids on the C-terminal side of the transmembrane region have been deleted.

4. The modified M2 polypeptide of claim 1, wherein the native M2 protein is from the A/Aichi/2/68 (H3N2) virus.

5. The modified M2 polypeptide of claim 4, wherein amino acids 26-43 have been deleted.

6. The modified M2 polypeptide of claim 4, wherein amino acids 26-55 have been deleted.

7. The modified M2 polypeptide of any one of claims 1 to 6, wherein the deleted amino acid residues are replaced one or more neutral or hydrophilic amino acid residues, provided that the total number of amino acid residues in the modified M2 polypeptide is less than or equal to the number in the native M2 polypeptide.

8. The modified M2 polypeptide of claim 7, wherein all of the deleted amino acids are replaced with from one to six glycine residues.

9. A modified M2 polypeptide with reduced hydrophobicity and enhanced recombinant expression relative to a native M2, the modified M2 polypeptide comprising a sequence of amino acids identical to a native M2 protein in which from one to all of the amino acid residues of the transmembrane region and from 0 to 12 amino acid residues adjacent to the transmembrane region on the C-terminal side are replaced with neutral or hydrophilic amino acid residues.

10. The modified M2 polypeptide of claim 9, wherein all of the amino acid residues of the transmembrane region have been substituted with neutral or hydrophilic residues.

11. The modified M2 polypeptide of claim 9, wherein all of the amino acid residues of the transmembrane region and from one to twelve amino acids adjacent to the transmembrane region on the C-terminal side have been substituted with neutral or hydrophilic residues.

12. The modified M2 polypeptide of any one of claims 9 to 11, wherein the native M2 protein is from the A/Aichi/2/68 (H3N2) virus.

13. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide according to and one of claims 1 to 12.

14. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to any one of claims 1 to12.

15. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 14.

16. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 15.

17. The host cell according to claim 16, wherein the host is a prokaryote.

18. The host cell according to claim 16, wherein the prokaryote is *E. coli*.

19. A composition comprising a modified M2 polypeptide of any one of claims 1 to 12 and a pharmaceutically acceptable carrier.

20. An antibody to a modified M2 polypeptide of any one of claims 1 to12.

21. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 20.

22. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to any one of claims 1 to 12 and detecting the binding of antibodies to the modified M2 protein.

23. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 19.

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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For Receiving Office use only

International Application No. PCT/US 98/16379

International Filing Date 03 SEP 1998 (06 08 1998) (03.09.98)

Name of receiving Office and "PCT International Application" PCT INTERNATIONAL APPLICATION

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 98,506-A

Box No. I TITLE OF INVENTION

PREPARATION AND USE OF RECOMBINANANT INFLUENZA A VIRUS M2 CONSTRUCTS AND VACCINES

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

Centers for Disease Control and Prevention
1600 Clifton Road, NE, Mailstop: G16
Atlanta, Georgia 30333
United States of America

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

FRACE, A. Michael
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United States of America

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: US

State *(i.e. country)* of residence: US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KLIMOV, Alexander I.
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[Russian Federation] US

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: RU

State *(i.e. country)* of residence: RU

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

KATZ, Jacqueline M.
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RO

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: AU

State *(i.e. country)* of residence: AU

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (i.e. country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

☒ **AP** **ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT

☒ **EA** **Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

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☒ **OA** **OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

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☒ **CZ** Czech Republic .....
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Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

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Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country *(in which, or for which, the application was filed)*	Filing Date *(day/month/year)*	Application No.	Office of filing *(only for regional or international application)*
item (1) US	06 August 1997 (06-08-97)	08/906,930	
item (2)	( )		
item (3)	( )		

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): 1 (one)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA/EP

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search, such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year):* Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	4 sheets
2. description	:	27 sheets
3. claims	:	6 sheets
4. abstract	:	1 sheets
5. drawings	:	4 sheets
Total	:	42 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☒ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☒ nucleotide and/or amino acid sequence listing (diskette)
8. ☒ other *(specify):* Sequence Listing (Paper Copy); Statement Under 1.822(F)

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Michael S. Greenfield

06 08' (03.09.98)

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1. Date of actual receipt of the purported international application: 01 Rec'd PCT/PTO 03 SEP 1998 '06 AUG
2. Drawings: ☐ received: ☐ not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA/EP
6. ☐ Transmittal of search copy delayed until search fee is paid

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Date of receipt of the record copy by the International Bureau: 22 SEPTEMBER 1998 (22.09.98)

PREPARATION AND USE OF RECOMBINANANT INFLUENZA A VIRUS M2 CONSTRUCTS AND VACCINES

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of vaccines against influenza A virus and the constructs useful in their production.

Description of the Related Art

The prior art illustrates the current strategy for control of influenza by yearly vaccination with whole-virus or subunit vaccines. The currently-licensed vaccines are designed to stimulate neutralizing antibodies against hemagglutinin (HA) and/or neuraminidase (NA), the major surface antigens of the influenza virus. However, due to frequent and unpredictable structural variation of HA and NA, influenza vaccines must be seasonally customized to circulating virus strains, a process which is deficient in providing protective immunity against all but closely matched viral strains.

There is a need for a vaccine subunit component capable of inducing broader, more cross-reactive immunity to type A influenza viruses. One such component may be M2, a structurally conserved influenza A viral surface protein (Slepushkin *et al.*, 1995; Ito *et al.*, 1991). The DNA sequences of the M2 genes of numerous influenza A viruses are known (Ito *et al.*, 1991). M2 is thought to provide an obligatory transmembrane proton flux for viral replication (Sugrue *et al.*, 1990; Ciampor *et al.*, 1992b; Grambas and Hay, 1992). As a membrane transport protein, M2 functions as an open pore which conducts cations in a nonselective manner (Tosteson *et al.*, 1994; Shimbo *et al.*, 1996). This conductance is thought to permeabilize host cells expressing recombinant M2 and may explain difficulties that others have had in achieving high levels of recombinant M2 expression in prokaryotic as well as eukaryotic systems (Guinea and Carrasco, 1996; Black *et al.*, 1993).

Antibody to M2 has been shown to restrict influenza virus replication in cell culture and in infected mice (Zebedee and Lamb (1988) and Treanor *et al.*, (1990). Full length M2 expressed in baculovirus has been shown to raise serum titers and stimulate T-cell responses in immunized animals (Katz, *et al.*, 1996). Further, vaccination of mice with recombinant full-length M2 has been shown to enhance viral clearance from infected lungs and to provide protection from lethal challenge with heterologous influenza A virus (Slepushkin *et al.*, 1995).

Since M2 is not expressed to any extent in virions (Zebedee & Lamb, 1988), the current whole virus or split-product influenza vaccine contains only minimal amounts of M2. To be useful as a component of a vaccine, M2 must be expressed and purified as a recombinant product. However, expression of full-length M2 has been shown to be detrimental to cell culture in prokaryotic and eukaryotic expression systems (Guinea and Carrasco, 1996; Black *et al.*, 1993). To date, expression of sufficient quantities of recombinant M2 for use in experimental studies can only be accomplished by culturing eukaryotic host cells in the presence of the irreversible M2 inhibitor, amantadine.

Wholly apart from the challenges in expression of recombinant M2, the hydrophobic nature of full-length M2 compromises the yield and purity of M2 preparations and necessitates the use of detergents or other agents to maintain M2 in a soluble form. Certain such solubilizing agents are not desirable constituents of vaccine formulations. The present invention solves this shortcoming in the prior art by providing a modified M2 protein with reduced hydrophobicity and concomitantly enhanced solubility characteristics relative to full-length M2.

SUMMARY OF THE INVENTION

The present invention solves the problems of the prior art approaches to recombinant M2 production by providing new recombinant forms of M2 whose structure has been modified to allow simple prokaryotic expression as a soluble, readily purified variant protein that retains antigenic and immunogenic properties. A preferred embodiment of the present invention provides a recombinant construct in which at least the entire portion of the transmembrane domain has been deleted. Alternatively, residues within the transmembrane domain may simply be altered, for example by substitution of hydrophilic or neutral amino acid residues for hydrophobic residues, in such a way as to (a) enhance expression of the protein in prokaryotic and/or eukaryotic systems relative to the native protein and/or (b) render the modified M2 protein more soluble in aqueous solutions relative to the native M2 protein. The terms M2 polypeptide and M2 protein are used interchangeably herein. The present invention further provides vaccines comprised of these new recombinant forms of M2 and to methods of prevention and treatment of influenza A virus infections.

The foregoing merely summarizes certain aspects of the invention and is not intended, nor should it be construed as limiting the invention in any manner. All patent applications,

patents, and other publications recited herein are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram of M2 and deletion constructs sM2 and ssM2;

Fig. 2 represents growth curves showing the time course of cell replication and expression of full-length and modified M2 polypeptides;

Fig. 3 (A) is a coomassie-stained SDS-PAGE gel; and

Fig. 3 (B) is a Western blot of fusion proteins containing modified M2 polypeptides.

Fig. 4 displays the results of vaccination with M2 constructs as described in Example 7.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention provides for a modified M2 protein comprising the native M2 protein in which at least the hydrophobic, transmembrane region is deleted or substituted with neutral or hydrophilic residues. We have discovered that such a modified M2 protein is more soluble in aqueous solution relative to its native M2 protein counterpart and in a form suitable for high-yield expression and purification. Moreover, as demonstrated herein, modified M2 proteins according to this aspect of the invention are both immunogenic and immunoprotective, making them suitable for use as vaccines.

For the purposes of this invention, the transmembrane region of M2 is defined generally as that portion of the M2 polypeptide which spans all or part of the lipid bilayer of the influenza A virus surface. Residues 26-43 of the native M2 of the A/Aichi/2/68 (H3N2) virus correspond to the transmembrane region, although the modified M2 of the present invention can be constructed to correspond to the native M2 protein of any strain. Those skilled in the art will appreciate that comparable regions of other influenza A viruses and newly emerging influenza A viruses will correspond to this general description of a transmembrane region and the present invention contemplates removal or alteration of sufficient residues within the transmembrane region to render said region functionally inactive and, preferably, to reduce overall hydrophobicity, thereby allowing for efficient expression and purification of modified M2 polypeptides following culture in prokaryotic and eukaryotic hosts.

As mentioned, the modified M2 proteins according to the invention manifest enhanced expression in host organisms compared to the expression level of the native M2

protein. Although the invention is not limited by any theory, enhanced expression may arise due to inactivation (or significantly diminution) of the ion channel activity of the modified M2 polypeptide, thereby decreasing the polypeptide's toxicity to the expressing host organism. Preferably, modified M2 proteins of the invention are capable of being expressed in a host organism at levels of 5-50 mg/l or at levels sufficient to produce a visible band on coomassie stained gel.

In a preferred embodiment, the modified M2 protein according to this aspect of the invention comprises a sequence of amino acids identical to a native M2 protein in which the transmembrane region and from zero to twelve amino acid residues adjacent to the transmembrane region on its C-terminus side have been deleted. By this is meant that the modified M2 protein comprises the portion of a native M2 protein on the N-terminal side of the transmembrane region fused to a portion of the native M2 protein from the C-terminal side of the transmembrane region. In another preferred embodiment, the modified M2 protein comprises a sequence of amino acids identical to the native M2 protein of the A/Aichi/2/68 (H3N2) virus in which residues 26 through anywhere from 43 to 55 have been deleted. In other words, this embodiment comprises the N-terminal 25 amino acid sequence fused at its C-terminus to the N-terminal amino acid of the C-terminal portion of the native M2 protein, wherein the C-terminal portion begins (at its N-terminal end) at one of amino acid numbers 44-55 of the native M2 protein. In a more preferred embodiment, the modified M2 protein comprises a sequence of amino acids identical to the native M2 protein of the A/Aichi/2/68 (H3N2) virus in which residues 26-43 have been deleted. In another more preferred embodiment, the modified M2 protein comprises a sequence of amino acids identical to the native M2 protein of the A/Aichi/2/68 (H3N2) virus in which residues 26-55 have been deleted.

In another embodiment, the deleted residues of the native M2 protein are replaced with one or more neutral or hydrophilic amino acids. In this embodiment, the number of amino acid residues in the modified M2 protein is less than or equal to the number in the native M2 protein. The deleted residues are preferably replaced with from one to six neutral or hydrophilic amino acid residues. In another preferred embodiment, the neutral or hydrophilic residues in the foregoing embodiments are glycine.

In another preferred embodiment, the modified M2 protein according to this aspect of the invention comprises a sequence of amino acids identical to the native protein in which from one to all of the amino acid residues of the transmembrane region and from zero to

twelve amino acid residues adjacent to the transmembrane region on its C-terminus side have been substituted with neutral or hydrophilic amino acids. In this embodiment, the modified M2 protein has the same number of amino acid residues as the native M2 protein. The number of amino acid substitutions and the type of substitution are sufficient to yield a protein having a higher solubility in aqueous solution than the native protein and generally increased expression in host organisms. There are numerous such proteins according to this embodiment, and it is but a routine matter for one of ordinary skill in the art to substitute one or more of the known hydrophilic and/or neutral amino acids into the transmembrane region and/or the region adjacent to it on the C-terminal side to obtain a modified M2 protein according to this embodiment of the invention. Preferably the modified M2 protein according to this aspect of the invention has, except for the substituted amino acids, a sequence identical to the native M2 of the A/Aichi/2/68 (H3N2) virus.

In another preferred embodiment, the modified M2 protein according to this aspect of the invention comprises any one of the previously recited embodiments in the form of a fusion protein. In one embodiment, the modified M2 protein is fused to a polypeptide that renders the fusion construct more easily purified than the modified M2 protein alone. In a preferred embodiment, the modified M2 protein is fused to the glutathione S-transferase (GST) (*e.g.*, from *Schistosoma japonicum*). Alternatively or additionally, the modified M2 protein can be fused to a signal peptide so as to direct secretion of the polypeptide from the expressing host cell. It is but a routine matter for those skilled in the art to identify, make, and use other fusion proteins according to the invention employing a wide variety of polypeptides.

Those skilled in the art will recognize that the modified M2 polypeptides of the present invention can be produced by any one of a variety of recombinant methods. The basic steps in the recombinant production of modified M2 polypeptides include:

a) construction of a synthetic or semi-synthetic DNA encoding the modified M2 polypeptide,

b) integrating said DNA into an expression vector in a manner suitable for the expression of the modified M2 polypeptide either alone or as a fusion protein,

c) transforming an appropriate eukaryotic or prokaryotic host cell with said expression vector,

d) culturing said transformed or transfected host cell, and

e) recovering and purifying the recombinantly produced modified M2 polypeptides.

For recombinant expression, the modified M2 coding sequence may be wholly synthetic, semi-synthetic or the result of modification of the native M2 gene sequence.

In another aspect, the invention provides synthetic genes, the *in vitro* or *in vivo* transcription and translation of which will result in the production of modified M2 polypeptides. Such genes are derived from the gene sequence of the native M2 protein and suitably modified to encode the particular modified M2 protein of which expression is desired. Genes according to this aspect may be constructed by techniques well known in the art. Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet definite number of DNA sequences may be constructed that encode modified M2 polypeptides. The gene encoding the modified M2 polypeptides may be created by synthetic methodology. Such methodology of synthetic gene construction is well known in the art. The DNA sequence corresponding to the modified M2 polypeptide genes can be generated using conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404).

One of ordinary skill in the art will recognize that the nucleotide sequence for the M2 gene from other isolates of influenza type A viruses will be very similar, though not necessarily identical, to the M2 gene sequence for A/Aichi/2/68 (H3N2). The teachings herein are readily applicable to closely related sequences from other influenza A type viruses, using techniques well established in the art. Accordingly, this invention contemplates variants of other M2 genes in which, as here, the transmembrane and/or hydrophobic regions have been deleted. Hybridization and wash conditions and protocols for obtaining sequences of a desired degree of homology are standard and well known to those skilled in the art. See, *e.g., Current Protocols in Molecular Biology*, vol. 1, unit 2.10 (John Wiley & Sons, Inc. 1997). These techniques can be employed on a routine basis to isolate homologous DNA molecules according to this aspect of the invention. Routine adjustment of the hybridization and wash conditions enable artisan of ordinary skill to obtain DNA of virtually any desired degree of homology. Nucleic acids according to this embodiment can be obtained using the protocol set forth in Chapter 5, Table 3 of *Nucleic Acid Hybridization: A Practical Approach* (Hames & Higgins, Eds., IRL Press, Washington D.C., 1985).

Vector Construction Generally

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

To effect the translation of the modified M2 polypeptides, one inserts the engineered modified M2 DNA coding sequence in any of a plethora of appropriate recombinant DNA expression vectors through the use of appropriate restriction endonucleases. A synthetic modified M2 coding sequence is designed to possess restriction endonuclease cleavage sites at either end of the transcript to facilitate isolation from and integration into these expression and amplification and expression plasmids. The coding sequence may be readily modified by the use of synthetic linkers to facilitate the incorporation of this sequence into the desired cloning vectors by techniques well known in the art. The particular endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the parent expression vector to be employed. The choice of restriction sites are chosen so as to properly orient the modified M2 coding sequence with control sequences to achieve proper in-frame reading and expression of the modified M2 polypeptide genes.

In general, plasmid vectors containing promoters and control sequences which are derived from species compatible with the host cell are used with these hosts. The vector ordinarily carries a replication site as well as marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar, *et al.*, 1977). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid must also contain or be modified to contain promoters and other control elements commonly used in recombinant DNA construction.

The modified M2 polypeptide coding sequence must be positioned so as to be in proper reading frame with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which the modified M2 polypeptide is to be expressed. In the preferred practice of the invention, the promoter-operator region is placed in the same sequential orientation with respect to the ATG start codon of DNA sequence encoding the modified M2 polypeptide as the promoter-operator occupies with respect to the ATG-start codon of the gene from which it was derived. Synthetic or modified promoter-operator regions such as the tac promoter are well known in the art. When employing such

synthetic or modified promoter-operator regions they should be oriented with respect to the ATG start codon of the modified M2 polypeptide coding sequence as directed by their creators.

Prokaryotic Expression

In general, prokaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, *E. coli* K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include *E. coli* B and *E. coli* X1776 (ATCC No. 31537), *E. coli*_W3110 (prototrophic, ATCC No. 27325), bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcescans*, and various pseudomonas species may be used. Promoters suitable for use with prokaryotic hosts include the b-lactamase (vector pGX2907 [ATCC 39344] contains the replicon and b-lactamase gene) and lactose promoter systems (Chang *et al.*, 1978; Goeddel *et al.*, 1979), alkaline phosphatase, the tryptophan (trp) promoter system (vector pATH1 [ATCC 37695] is designed to facilitate expression of an open reading frame as a trpE fusion protein under control of the trp promoter) and hybrid promoters such as the tac promoter (isolatable from plasmid pDR540 ATCC-37282). However, other functional bacterial promoters, whose nucleotide sequences are generally known, enable one of skill in the art to ligate them to DNA encoding modified M2 polypeptides using linkers or adapters to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno sequence operably linked to the DNA encoding modified M2 polypeptides. These examples are illustrative rather than limiting.

Fusion Proteins

The modified M2 polypeptides may be made either by direct expression or as fusion protein comprising the modified M2 polypeptide followed by enzymatic or chemical cleavage. It is often observed in the production of certain peptides in recombinant systems that expression as a fusion protein prolongs the lifespan and/or increases the yield of the desired peptide. A variety of peptidases (*e.g.*, trypsin) which cleave a polypeptide at specific sites or digest the peptides from the amino or carboxy termini (*e.g.*, diaminopeptidase) of the peptide chain are known. Furthermore, particular chemicals (*e.g.*, cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding

sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. See e.g., Carter P. (1990).

Therefore, it may be desirable to fuse the coding sequence of a particular modified M2 polypeptide in-frame to a larger gene coding sequence resulting in the production of a fusion protein.

Eukaryotic Expression

The modified M2 polypeptides can also be recombinantly produced in eukaryotic expression systems.

Eukaryotic Signal Peptides

An advantage of eukaryotic expression systems is that it is possible to obtain a secreted protein product. If such a result is desired, it is necessary to modify the coding sequence of the modified M2 polypeptide to incorporate a translated signal peptide encoding sequence. Generally, signal peptides are proteolytically cleaved from a residual protein as part of the secretory process in which the protein is transported into the host cell periplasm or culture medium.

It is well known in the art that signal peptides facilitate the extracellular discharge of secretory proteins in both prokaryotic and eukaryotic environments. It has been shown that the addition of a heterologous signal peptide to a normally cytosolic protein will result in the extracellular transport of the normally cytosolic protein in *E. coli.* (MacIntyre, *et al.,* 1987). It is well known in the art that alternate signal peptide sequences may function with heterologous coding sequences. The recombinant production of such fusion proteins maybe accomplished by the addition of a DNA sequence encoding a signal peptide appropriate to the host organism inserted 5′ to, and in reading frame with, the protein coding sequence.

Signal peptides are well known in the art which could be similarly incorporated into the modified M2 polypeptide structure. In the preferred practice of the invention the signal peptide used is a signal peptide native to a secretory protein of the host cell line. Furthermore, the signal sequence may be wholly synthetic. Synthetic "idealized" signal peptides have been shown to function in both prokaryotic and eukaryotic environments. (von Heijne, G.,1990). The principles of signal peptides are similar in both prokaryotic and eukaryotic organisms. Both prokaryotic and eukaryotic signal peptides possess an overall three domain structure and with no precise sequence conservation necessary to preserve function. (von Heijne, G., *supra*). Generally, the presence of basic and/or charged amino

acid residues near the amino terminus of the structural protein inhibits secretion. (Yamane, K., *et al.*, 1988; Summers, R.G., *et al.*, 1989). In order to ensure the efficient cleavage of the signal peptide from the fusion protein construct, it is desirable to maintain the nature of the amino acid sequence at the interface between the signal peptide and the coding sequence of the mature art protein. Conservation of charge and hydrophobicity and the elimination of charged residues immediately downstream of the signal peptide cleavage point are generally important to efficient translocation. However, it is not critical that any one particular amino acid sequence be maintained.

Eukaryotic Promoters

Preferred promoters controlling transcription in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, *e.g.*, b-actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. (Fiers, *et al.*, 1978). The entire SV40 genome may be obtained from plasmid pBRSV, ATCC 45019. The immediate early promoter of the human cytomegalovirus may be obtained from plasmid pCMBb (ATCC 77177). Of course, promoters from the host cell or related species also are useful herein.

Eukaryotic Enhancers

Transcription of a DNA encoding modified M2 polypeptides by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5′ (Laimins, L. *et al.*, 1981) and 3′ (Lusky, M. L., *et al.*, 1983) to the transcription unit, within an intron (Banerji, J. L. *et al.*, 1983) as well as within the coding sequence itself (Osborne, T. F., *et al.*, 1984). Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, a-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Eukaryotic Expression Vectors

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding modified M2 polypeptides. The 3' untranslated regions also include transcription termination sites.

Eukaryotic Selectable Markers

Expression vectors may contain a selection gene, also termed a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR, which may be derived from the BglII/HindIII restriction fragment of pJOD-10 [ATCC 68815]), thymidine kinase (herpes simplex virus thymidine kinase is contained on the BamHI fragment of vP-5 clone [ATCC 2028]) or neomycin (G418) resistance genes (obtainable from pNN414 yeast artificial chromosome vector [ATCC 37682]). When such selectable markers are successfully transferred into a mammalian host cell, the transfected mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow without a supplemented media. Two examples are: CHO DHFR$^-$ cells (ATCC CRL-9096) and mouse LTK$^-$ cells (L-M(TK-) ATCC CCL-2.3). These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nutrients are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin (Southern P. and Berg, P., 1982), mycophenolic acid (Mulligan, R. C. and Berg, P. 1980) or hygromycin (Sugden, B. *et al.*, 1985). The three examples given above employ bacterial genes under eukaryotic control to convey resistance

to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively.

Eukaryotic Host Cells

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as Maniatis, *et al.* (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York or Current Protocols in Molecular Biology (1989) and supplements.

Preferred suitable host cells for expressing the vectors of this invention encoding modified M2 polypeptides in higher eukaryotes include: African green monkey kidney line cell line transformed by SV40 (COS-7, ATCC CRL-1651); transformed human primary embryonal kidney cell line 293,(Graham, F. L. *et al.* 1977); baby hamster kidney cells (BHK-21(C-13), ATCC CCL-10); chinese hamster ovary cells CHO-DHFR$^-$ (ATCC CRL-9096), mouse Sertoli cells (TM4, ATCC CRL-1715); African green monkey kidney cells (VERO 76, ATCC CRL-1587); human cervical epitheloid carcinoma cells (HeLa, ATCC CCL-2); canine kidney cells (MDCK, ATCC CCL-34); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); human diploid lung cells (WI-38, ATCC CCL-75); human hepatocellular carcinoma cells (Hep G2, ATCC HB-8065);and mouse mammary tumor cells (MMT 060562, ATCC CCL51).

Yeast Expression

In addition to prokaryotes, eukaryotic microbes such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb, *et al.*, 1979); Kingsman *et al.*, 1979); Tschemper *et al.*, 1980) is commonly used. This plasmid already contains the trp gene which provides a selectable marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, 1977).

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD ATCC 53231 and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 ATCC 39532), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 ATCC 57090, 57091), *Zymomonas mobilis* (United States Patent No. 5,000,000 issued March 19, 1991), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV ATCC 39475, United States Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose (GAL1 found on plasmid pRY121 ATCC 37658) utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal from *Saccharomyces cerevisiae* (found in conjuction with the CYC1 promoter on plasmid YEpsec--hI1beta ATCC 67024), also are advantageously used with yeast promoters.

Expression In Vaccinia

The modified M2 polypeptides may also be expressed in vaccinia virus.

Paoletti, *et al.* (U.S. Patent Nos. 4,722,848 and 5,110,587) describe a general method wherein exogenous DNA sequences are introduced into nonessential regions of the vaccinia virus genome, thereby effecting expression of said exogenous sequences. Paoletti, *et al.* (U.S. Patent No. 5174,993) describes a method for inducing an immunological response in a mammal to a pathogen by incorporation of exogenous DNA sequences derived from the pathogen into avipox virus. The teachings of these patents are hereby incorporated in their entirety be reference. The method of these patents may readily be modified to incorporate modified M2 polypeptide sequences of the present invention.

Expression By Naked DNA

The modified M2 polypeptides may also be expressed *in vivo* using the "naked DNA" approach as described by Felgner, *et al.* (U.S. Patent No. 5,589,466, 5,703,055, and

5,580,859). This approach entails delivery (typically by injection) of isolated nucleic acids into mammalian tissue, resulting in transient expression of the injected nucleic acids. Transient expression of foreign genes in mammalian tissue invokes an immune response which can be protective. The teachings of this patent are hereby incorporated in their entirety by reference and may readily be modified for use with the modified M2 polypeptide sequences of the present invention.

In another aspect, the invention comprises immunogenic compositions, including vaccines. Such immunogenic compositions comprise a modified M2 protein according to the first aspect of the invention and may be prepared as injectables, as liquid solutions, suspensions or emulsions. The active immunogenic ingredient or ingredients may be mixed with pharmaceutically acceptable excipients which are compatible therewith. Such excipients may include water, saline, dextrose, glycerol, ethanol, and combinations thereof. The immunogenic compositions and vaccines may further contain auxiliary substances, such as wetting or emulsifying agents, pH buffering agents, or adjuvants to enhance the effectiveness thereof. Immunogenic compositions and vaccines may be administered parenterally, by injection subcutaneously or intramuscularly. Alternatively, the immunogenic compositions formed according to the present invention, may be formulated and delivered in a manner to evoke an immune response at mucosal surfaces. Thus, the immunogenic composition may be administered to mucosal surfaces by, for example, the nasal or oral (intragastric) routes. Alternatively, other modes of administration including suppositories and oral formulations may be desirable. For suppositories, binders and carriers may include, for example, polyalkalene glycols or triglycerides. Such suppositories may be formed from mixtures containing the active ingredient(s) in the range of about 0.5 to about 10%, preferably about 1 to 2%. Oral formulations may include normally employed incipients such as, pharmaceutical grades of saccharine, cellulose and magnesium carbonate. These compositions can take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain about 1 to 95% of the active ingredient(s), preferably about 20 to about 75%.

The immunogenic preparations and vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective, protective and immunogenic. The quantity to be administered depends on the subject to be treated, including, for example, the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, and if needed, to produce a cell-mediated

immune response. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner. However, suitable dosage ranges are readily determinable by one skilled in the art and may be of the order of micrograms of the active ingredient(s) per vaccination. Suitable regimes for initial administration and booster doses are also variable, but may include an initial administration followed by subsequent administrations. The dosage may also depend on the route of administration and will vary according to the size of the host.

The concentration of the active ingredient protein in an immunogenic composition according to the invention is in general about 1 to 95%. A vaccine which contains antigenic material of only one pathogen is a monovalent vaccine. Vaccines which contain antigenic material of several pathogens are combined vaccines and are also contemplated by the present invention. Such combined vaccines contain, for example, material from various pathogens or from various strains of the same pathogen, or from combinations of various pathogens.

Immunogenicity can be significantly improved if the antigens are co-administered with adjuvants, commonly used as 0.05 to 0.1 percent solution in phosphate-buffered saline. Adjuvants enhance the immunogenicity of an antigen but are not necessarily immunogenic themselves. Adjuvants may act by retaining the antigen locally near the site of administration to produce a depot effect facilitating a slow, sustained release of antigen to cells of the immune system. Adjuvants can also attract cells of the immune system to an antigen depot and stimulate such cells to elicit immune responses.

Immunostimulatory agents or adjuvants have been used for many years to improve the host immune responses to, for example, vaccines. Intrinsic adjuvants, such as lipopolysaccharides, normally are the components of the killed or attenuated bacteria used as vaccines. Extrinsic adjuvants are immunomodulators which are typically non-covalently linked to antigens and are formulated to enhance the host immune responses. Thus, adjuvants have been identified that enhance the immune response to antigens delivered parenterally. Some of these adjuvants are toxic, however, and can cause undesirable side-effects, making them unsuitable for use in humans and many animals. Indeed, only aluminum hydroxide and aluminum phosphate (collectively commonly referred to as alum) are routinely used as adjuvants in human and veterinary vaccines. The efficacy of alum in increasing antibody responses to diphtheria and tetanus toxoids is well established and a HBsAg vaccine has been adjuvanted with alum. A wide range of extrinsic adjuvants can provoke potent immune responses to antigens. These include saponins complexed to membrane protein antigens

(immune stimulating complexes), pluronic polymers with mineral oil, killed mycobacteria in mineral oil, Freund's complete adjuvant, bacterial products, such as muramyl dipeptide (MDP) and lipopolysaccharide (LPS), as well as lipid A, and liposomes.

To efficiently induce humoral immune responses (HIR) and cell-mediated immunity (CMI), immunogens are often emulsified in adjuvants. Many adjuvants are toxic, inducing granulomas, acute and chronic inflammations (Freund's complete adjuvant, FCA), cytolysis (saponins and pluronic polymers) and pyrogenicity, arthritis and anterior uveitis (LIPS and MDP). Although FCA is an excellent adjuvant and widely used in research, it is not licensed for use in human or veterinary vaccines because of its toxicity.

Desirable characteristics of ideal adjuvants include:

(1) lack of toxicity;

(2) ability to stimulate a long-lasting immune response;

(3) simplicity of manufacture and stability in long-term storage;

(4) ability to elicit both CMI and HIR to antigens administered by various routes, if required;

(5) synergy with other adjuvants;

(6) capability of selectively interacting with populations of antigen presenting cells (APC);

(7) ability to specifically elicit appropriate T_H1 or T_H2 cell-specific immune responses; and

(8) ability to selectively increase appropriate antibody isotype levels (for example, IgA) against antigens.

US Patent No. 4,855,283 granted to Lockhoff et al on August 8, 1989 which is incorporated herein by reference thereto teaches glycolipid analogues including N-glycosylamides, N-glycosylureas and N-glycosylcarbamates, each of which is substituted in the sugar residue by an amino acid, as immuno-modulators or adjuvants. Thus, Lockhoff et al. (US Patent No. 4,855,283 and ref. 32) reported that N-glycolipid analogs displaying structural similarities to the naturally-occurring glycolipids, such as glycosphingolipids and glycoglycerolipids, are capable of eliciting strong immune responses in both herpes simplex virus vaccine and pseudorabies virus vaccine. Some glycolipids have been synthesized from long-chain-alkylamines and fatty acids that are linked directly with the sugars through the anomeric carbon atom, to mimic the functions of the naturally occurring lipid residues.

U.S. Patent No. 4,258,029 teaches that octadecyl tyrosine hydrochloride (OTH) functioned as an adjuvant when complexed with tetanus toxoid and formalin inactivated type I, II and III poliomyelitis virus vaccine. Also, Nixon-George *et al.* reported that octadecyl esters of aromatic amino acids complexed with a recombinant hepatitis B surface antigen, enhanced the host immune responses against hepatitis B virus.

The choice of adjuvant or combination of adjuvants is entirely within the skills of the ordinarily skilled immunologist. The adjuvants discussed above included adjuvants useful in experimental settings as well as adjuvants of potential human or veterinary application. The influenza A vaccines of the invention can be formulated using any of the aforementioned adjuvants and as such the use of any of the adjuvants in combination or in conjunction with the modified M2 polypeptides of the invention is contemplated by and is thus within the scope of the present invention.

In another aspect, the invention comprises methods of generating antibodies to M2 and the antibodies thereby produced, the method comprising administering to a subject capable of producing antibodies to M2 a composition according to the invention (which composition comprises a modified M2 protein according to the invention) and collecting antibodies to M2 from the subject.

In another aspect, the invention provides methods for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to the first aspect of the invention and detecting the binding of antibodies to the modified M2 protein. The presence of antibodies indicates current or previous exposure of the subject to influenza.

In another aspect, the invention provides methods for limiting viral influenza A infection in a subject, the method comprising administering to the subject a prophylactically effective amount of a modified M2 protein-containing composition according to the invention. Prophylactic amounts of the composition can be determined routinely.

In yet another aspect, the invention provides methods of treating a subject suffering from viral influenza A infection, the method comprising administering to the subject a therapeutically effective amount of the

The following examples are provided for illustrative purposes only and are not intended, nor should they be construed, as limiting the invention in any manner.

EXAMPLES

Example 1

PCR and plasmid construction

Full length and truncated forms of M2 cDNA were made by PCR from RNA of A/Aichi/2/68 (H3N2) virus. Figure 1 shows schematic diagrams of M2 and the deletion constructs sM2 and ssM2. Each diagram shows a boxed diagram of the M2 structure and the area deleted. Below each are the oligonucleotide primer positions used in constructing the cDNAs. F(1) is forward primer 1, F(2) forward primer 2, R(1) reverse primer 1, (R2) reverse primer 2. (A) The M2 amino acid sequence is represented in three boxes, an extracellular domain, a darkened transmembrane domain, and an intracellular domain. Notations within these boxes include epitopes or post-translational modifications which have been described for M2: (<>) epitope for Mab 14C2; vertical dashed lines are cysteine sulfhydryl linkages; (p) is a palmitoylation site; and (*) is a phosphorylation site. (B) sM2 shows a deletion between Pro(25) and Asp(44). The deletion is performed with primer annealment indicated with vertical lines. ssM2 has a deletion between Pro(25) to Glu(56).

Four oligonucleotide primers were designed to generate cDNA. Forward-1 primer (5′-CCCGAATTCTTATGAGCCTTCTAACCGAGGTCGAAACGCCTATCAGAAACGA-ATGGGGATGC-3′) (SEQ ID NO: 1) was specific for the 5′ coding region of the M2 gene (nucleotides 1-51) and began with a 5′ *EcoR1* restriction site. The reverse-1 primer (5′-GTCTTTGCTTACCCCTACGTCTACGTTGCTAAGTTCACTAGGACCTCCTCCC-3′) (SEQ ID NO: 2) [3′-CCCTCCTCCAGGATCACTTGAATCGTTGCATCTGCATCCCC-ATTCGTTTCTG-5′] coded for 3′ amplification from nucleotide 75. The forward-2 primer (sM2, 5′-CAAGTGATCCTGGAGGAGGAGATCGTCTCTTCTTCAAATGC-3′ (SEQ ID NO: 3); ssM2, 5′-CAAGTGATCCTGGAGGAGGAAAACACGGTCTGAAAAGAGGGCC-3′ (SEQ ID NO: 5)) was varied to flank areas chosen for deletion and contained a 5′ region homologous to the reverse-1 primer to allow annealing. These primers also coded for three glycine residues inserted in place of the deleted segments. The reverse-2 primer (3′-CTATCAGTAAAGCAGTCGTATCTCGACC-TCATCAGCTGCCC-5′) (SEQ ID NO: 4) [5′-CCCGTCGACTACTCCAGCTCTATGCTGACGAAATGAC-TATC-3′] coded for the 3′ end of M2 and provided a 3′ *SalI* restriction site. Full-length M2 cDNA was prepared by RT-PCR using forward -1 and reverse -2 primers. For deletion constructs, "5′ side" and "3′ side" reactions were carried out, annealed, then amplified to produce full length M2 or

deletion cDNAs. These were digested with *EcoR1* and *Sal1*, purified by gel electrophoresis and ligated into *EcoR1* and *Sal1* sites of a plasmid vector, pGEX-5X$_a$ (Pharmacia, Piscataway, NJ). The construct which has been designated sM2 has a deletion between amino acids 25 and 44 of native M2. The construct designated ssM2 has a deletion between amino acids 25 and 56 of native M2. Plasmids were transformed into competent *E. coli* strain JM109 (Stratagene, La Jolla, CA). Plasmid sequences were verified by automated nucleotide sequence analysis using standard protocols.

Example 2

Expression and isolation of fusion protein

The pGEX vector (Pharmacia, Piscataway, NJ) was chosen to express the constructs, and allows purification of the products with a simple affinity matrix. pGEX is designed to express, under control of the inducible *tac* promoter, glutathione S-transferase (GST; from *Schistosoma japonicum*) as a 29 kDa fusion to the N-terminus of a subcloned sequence (Smith and Johnson, 1988). The fusion protein can be purified from bacterial lysates by affinity chromatography using glutathione sepharose® 4B. The fusion product may also be separated by a site-specific protease, Factor Xa, whose site is immediately downstream of the C-terminus of the GST.

Soluble fusion proteins

Cells were grown from frozen stocks in overnight cultures of Luria broth (LB) containing 100 μg/ml ampicillin. This culture was then diluted 1:10 the next morning and grown for 1.5 hr at 37°C with vigorous shaking. IPTG (isopropyl β-D-thiogalactoside) was then added to a final concentration of 0.1 mM and incubation continued for 3-4 hrs. To monitor cell growth an aliquot of culture was taken every 0.5 hr after the initial dilution and cell density ($A_{600\ nm}$) was measured over the induction period. Cells were pelleted by centrifugation and resuspended in cold lysis buffer (50 Tris, 100 NaCl, 1 EDTA, pH 8.0). Lysozyme was added to 1 mg/ml and phenylmethylsulfonylfluoride (PMSF) added to a concentration of 0.5 mM. The suspension was kept on ice for 15 min. Dithiothreitol (DTT) was added to a concentration of 5 mM, and the suspension was lysed by sonication (probe-tip) on ice for 1 min. Triton X-100 was added to a concentration of 1% and the lysate was mixed gently for 0.5 hr. The lysate was then centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was decanted and added to a washed glutathione sepharose® (Pharmacia, Piscataway, NJ) slurry (50% v/v in phosphate-buffered saline (PBS)), with the slurry volume

being equal to 0.2% of the original bacterial culture volume. This mixture was gently stirred for 30 min. The sepharose® was then pelleted, the supernatant removed and discarded. The sepharose® was washed a minimum of three times in PBS. Fusion protein was eluted from the sepharose® pellet by addition of elution buffer (50 Tris, 10 reduced glutathione, pH 8.0) using a volume equal to the bed volume of sepharose®. The elution buffer/resin mixture was mixed for 15 min at room temperature, then pelleted at 500 x *g* for 5 min. The supernatant was harvested and a second elution was performed for residual product.

To enzymatically cleave ssM2 from the GST moiety the fusion product was left bound to sepharose® and treated overnight with 10 μg of Factor Xa (New England Biolabs, Beverly, MA) at 4°C. The sepharose® with bound GST was spun down, and the supernatant, containing the released ssM2, was harvested.

Insoluble fusion proteins

The insoluble forms of M2 were isolated using the above protocol with the suggested additions of Frangioni and Neel (1993), which include: (1) introduction of 1.5% sarkosyl prior to sonication and (2) raising the concentration of Triton X-100 to 4%. PBS washes of the bound glutathione sepharose® and the elution buffer contained 0.1% Triton X-100.

Example 3

Electrophoresis and western blotting

Expressed proteins were analyzed for size and purity on an SDS-12%- polyacrylamide gel, followed by staining with Coomassie brilliant blue R-250. Figure 3 (A) shows an SDS-PAGE gel of recombinant proteins. Lanes are: (1) molecular weight markers (2) a sample from the crude bacterial lysate of an induced sM2/G culture (3) a purified sample of GST protein (4) sM2/G protein (6) ssM2 protein which is isolated by cleaving ssM2/G with factor Xa protease. Molecular weights were compared to low molecular weight Rainbow Markers (Amersham International, Arlington Heights, IL.). For immunoblotting, gels were transferred to Immobilon-P membrane (Millipore, Bedford, MA) using a semi-dry transblot apparatus (Bio-Rad, Richmond, CA). Membranes were immunoblotted with a 1:5000 dilution of ascitic fluid containing the M2-specific antibody 14C2, followed by labeling with the ECL system (Amersham International, Arlington Heights, IL.) and exposure to X-ray film. Figure 3 (B) shows a Western blot of GST, sM2/G and ssM2/G using 14C2 as the primary antibody.

Example 4

Animal vaccination and challenge

Fusion proteins or control GST protein were added to equal volumes of PBS and incomplete Freund's adjuvant. A volume of 0.2 ml, containing 10 μg of protein was injected intraperitoneally (i.p.) into female BALB/c mice, aged 6-12 weeks. Boosts were given after 3 and 6 weeks for a total of 3 inoculations. Animals were bled from the orbital plexus at weeks 6 and 9 and individual sera were tested for antibodies which would react with a synthetic peptide composed of the first 17 amino acids of M2 (peptide PM_2-1, Slepushkin *et al.*, 1995). Antibody binding was detected on peptide-coated ELISA plates by adding horseradish peroxidase-conjugated anti-mouse Ig and *o*-phenylenediaminehydrochloride and hydrogen peroxide as colorimetric substrates. Titers are expressed as the highest dilution which yielded an optical density $(OD)_{490}$ two times higher than a similarly diluted control sera.

Following inoculations, mice were subjected to sub-lethal challenge by heterologous influenza A virus. Mice were anesthetized with CO_2 and were infected intranasally (i.n.) with 100 mouse infectious doses $(MID)_{50}$ of MA A/Ann Arbor/6/60 (H2N2) virus or A/Taiwan/1/86 (H1N1) [equivalent to5.3×10^6 and 1.3×10^5 egg infectious doses $(EID)_{50}$ respectively] in a volume of 50 μl of PBS. Mice were euthanized seven days after challenge. Lung homogenates were prepared and titrated into embryonated eggs for virus infectivity. Statistical significance of the data was determined using the Fisher exact test or Student's *t* test.

Example 5

Expression of recombinant M2 and M2 transmembrane deletants

The effects of expression of GST-fusion M2/G, sM2/G, ssM2/G, and GST on cell viability was tested at various times following induction of *E. coli* JM109 cells containing the respective pGEX constructs (Fig.2). Overnight cultures were diluted 1:100, reamplified for 2 hrs, and then induced with IPTG. At the time of induction the density of the M2/G culture was consistently lower than other cultures, presumably due to basal expression of the protein. The density of cells expressing full-length M2/G rose only marginally after induction, consistent with the reported lytic properties of M2 when expressed in *E coli* (Guinea and Carrasco, 1994). Addition of 5 μM amantadine to the culture media did not accelerate this growth pattern, and little, if any, M2/G fusion protein was obtainable from these cultures. In comparison, cultures of sM2/G and ssM2/G maintained a robust pattern of growth comparable to that of the control culture and the GST control protein.

The first deletion construct, which removes the transmembrane domain of M2 (sM2/G, residues 26-43), although expressing well, yielded no purified fusion protein with a standard lysis protocol, suggesting that it remained insoluble or in aggregated form. Adopting the sarkosyl protocol produced a modest yield of ~3 mg of fusion protein/L of bacterial culture, as determined by Bradford protein assay. Further deletion, from residue 26 to 55 (ssM2/G), was found to substantially improve fusion protein yield without the sarkosyl procedure, suggesting a soluble product. Residues 44-55 of mature M2 are all hydrophobic and contribute to a positive hydrophobic index for M2 in Kyte-Doolittle analysis (Lamb *et al.*, 1985). Values of up to 15 mg/L of culture are routinely achieved, with the purity of the fusion proteins in either preparation being >90%.

Example 6

SDS-PAGE and Western Blot

GST and fusion proteins were electrophoresed on polyacrylamide gels and either stained with Coomassie blue or prepared for immunoblotting. In Fig. 3 (A) a Coomassie stained gel is shown. A total protein sample from induced cells taken in the initial phase of the lysate is included (lane 2). GST (lane 3), from an induction of pGEX without M2 insert, is observed as a 29 kDa protein. Fusion proteins, sM2/G (lane 4) and ssM2/G (lane 5) are found at approximately 43 kDa. Panel (B) shows proteins run on a gel simultaneously with (A) , transferred to Immobilon-P membrane, and immunoblotted with M2-specific antibody 14C2. Proteins with an approximate weight of 43 kDa reacted with 14C2. GST is not visualized in the blot. Together, these results suggest that the 14C2 antibody epitope of the M2 deletion proteins is not obstructed by the fusion construction and that 14C2 is binding exclusively to the M2 domain.

Example 7

Immunogenic and protective properties of recombinant fusion proteins

The immunogenicity and protective efficacy of several M2 constructs were tested by vaccinating groups of BALB/c mice with sM2/G, ssM2/G, and enzymatically isolated ssM2 recombinant proteins. GST peptide was administered as a control. Results are shown in Table 1.

Table 1

Virus challenge of mice vaccinated with M2 constructs

Expt.	Vaccine	n	Serum antibody titer	Lung virus titer
1	GST	7	< 50	6.5±0.6 (AA/60)
2	GST	5	< 50	6.6±0.9 (AA/60)
3	PBS	5	< 50	6.8±0.4 (AA/60)
1	sM2/G	7	152,054	2.6±0.6 (AA/60)
2	sM2/G	5	19,390	2.2±1.5 (AA/60)
2	ssM2/G	5	11,138	2.9±1.4 (AA/60)
3	ssM2	7	123,838	3.2±0.7 (AA/60)
4	PBS	4	< 50	7.4±0.7 (AA/60)
4	GST	5	< 50	6.4±0.6 (AA/60)
4	PBS	3	< 50	7.0±0.6 (TW/86)
4	GST	6	< 50	7.0±1.0 (TW/86)
4	sM2/G	5	131,825	3.0±0.8 (AA/60)
4	sM2/G	6	131,825	3.7±0.5 (TW/86)
4	ssM2	5	1,202,264	3.2±1.2 (AA/60)
4	ssM2	6	1,202,264	3.6±0.5 (TW/86)

Challenge viruses were mouse adapted A/AA/6/60 (H2N2) and A/Taiwan/1/86 (H1N1).

All vaccinations were 3 x 10 μg of antigen, in Incomplete Freund's adjuvant.

[a] titers are expressed as the highest dilution of sera having a mean (OD_{490} greater than the mean plus two standard deviations of similarly diluted control sera.

[b] Mean $Log_{10}EID_{50}$/ml ± SD. Values for all virus titer reductions were significantly lower than control groups by Students *t*-test ($p<0.001$).

Groups of 5 to 7 mice were vaccinated as described above and analyzed for serum antibody which could recognize a synthetic peptide designed to mimic the extracellular domain of M2. Sera from mice vaccinated with GST control peptide showed no detectable (<50) antibody titer in any samples prior to challenge. However, groups vaccinated with sM2/G, ssM2/G, and ssM2 proteins showed elevated titers after two inoculations and a third

inoculation boosted the mean serum antibody titers for each group substantially. Surprisingly, deletion of the transmembrane region and additional hydrophobic residues of M2 does not appear to alter the immunological properties of the variant polypeptide, making such variant polypeptides suitable candidates for vaccines.

For virus challenge, mice vaccinated with either GST, sM2/G, ssM2/G, or ssM2 were challenged with heterologous MA A/Ann Arbor/6/60 (H2N2) or A/Taiwan/1/86 (H1N1) virus 4 weeks after their final boost. After 7 days the mice were euthanized, and lungs were harvested. Lung virus titers for the sM2/G and ssM2/G fusion protein groups, and isolated ssM2, were over 1000-fold lower than the PBS or GST protein control groups (Table 2). No significant difference was found between the sM2/G, ssM2/G, and ssM2 protective effect. This trend demonstrates a potential of the altered M2 peptides to protect against virus challenge, as has been shown for full-length baculovirus M2. Immunogenic compositions, suitable to be used as vaccines, may be prepared from immunogenic M2 as disclosed herein. Preferably, the antigenic material is extensively dialyzed to remove undesired small molecular weight molecules and/or lyophilized for more ready formulation into a desired vehicle. The immunogenic composition elicits an immune response which produces antibodies, including M2 antibodies which may inhibit viral replication, and also influenza A virus-specific cell-mediated immune responses.

The protocols and discussion provided above are sufficient to enable skilled workers in the field to reproduce the claimed inventions. The present inventors supplement the disclosure by listing scientific publications pertinent to the protocols and materials used to convey the present invention.

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We claim:

1. A modified M2 polypeptide with reduced hydrophobicity and enhanced recombinant expression relative to a native M2, the modified M2 polypeptide comprising a sequence of amino acids identical to a native M2 protein in which the transmembrane region and from 0 to 12 amino acid residues adjacent to the transmembrane region on the C-terminal side have been deleted.

2. The modified M2 polypeptide of claim 1, wherein the transmembrane region and none of the adjacent residues on the C-terminus side of the transmembrane region have been deleted.

3. The modified M2 polypeptide of claim 1, wherein the transmembrane region and the adjacent 12 amino acids on the C-terminal side of the transmembrane region have been deleted.

4. The modified M2 polypeptide of claim 1, wherein the native M2 protein is from the A/Aichi/2/68 (H3N2) virus.

5. The modified M2 polypeptide of claim 4, wherein amino acids 26-43 have been deleted.

6. The modified M2 polypeptide of claim 4, wherein amino acids 26-55 have been deleted.

7. The modified M2 polypeptide of any one of claims 1 to 6, wherein the deleted amino acid residues are replaced one or more neutral or hydrophilic amino acid residues, provided that the total number of amino acid residues in the modified M2 polypeptide is less than or equal to the number in the native M2 polypeptide.

8. The modified M2 polypeptide of claim 7, wherein all of the deleted amino acids are replaced with from one to six glycine residues.

9. A modified M2 polypeptide with reduced hydrophobicity and enhanced recombinant expression relative to a native M2, the modified M2 polypeptide comprising a sequence of amino acids identical to a native M2 protein in which from one to all of the amino acid residues of the transmembrane region and from 0 to 12 amino acid residues adjacent to the transmembrane region on the C-terminal side have substituted with neutral or hydrophilic amino acid residues.

10. The modified M2 polypeptide of claim 9, wherein all of the amino acid residues of the transmembrane region have been substituted with neutral or hydrophilic residues.

11. The modified M2 polypeptide of claim 9, wherein all of the amino acid residues of the transmembrane region and from one to twelve amino acids adjacent to the transmembrane region on the C-terminal side have been substituted with neutral or hydrophilic residues.

12. The modified M2 polypeptide of any one of claims 9 to 11, wherein the native M2 protein is from the A/Aichi/2/68 (H3N2) virus.

13. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide fusion protein according to and one of claims 1 to 6.

14. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide fusion protein according to claim 7.

15. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide fusion protein according to claim 8.

16. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide fusion protein according to and one of claims 9 - 11.

17. A modified M2 polypeptide fusion protein comprising a modified M2 polypeptide fusion protein according to claim 12.

18. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to any one of claims 1 to 6.

19. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to claim 7.

20. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to claim 8.

21. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to any one of claims 9 to 11.

22. A DNA molecule comprising a sequence of nucleotides encoding a modified M2 polypeptide according to claim 12.

23. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 18.

24. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 19.

25. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 21.

26. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 22.

27. A vector capable of expressing a modified M2 polypeptide, the vector comprising the DNA molecule of claim 22.

28. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 23.

29. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 24.

30. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 25.

31. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 26.

32. A host cell capable of expressing a modified M2 polypeptide, the host cell comprising a vector according to claim 27.

33. The host cell according to claim 28, wherein the host is a prokaryote.

34. The host cell according to claim 29, wherein the host is a prokaryote.

35. The host cell according to claim 30, wherein the host is a prokaryote.

36. The host cell according to claim 31, wherein the host is a prokaryote.

37. The host cell according to claim 28, wherein the prokaryote is *E. coli*.

38. The host cell according to claim 29, wherein the prokaryote is *E. coli*.

39. The host cell according to claim 30, wherein the prokaryote is *E. coli*.

40. The host cell according to claim 31, wherein the prokaryote is *E. coli*.

41. The host cell according to claim 32, wherein the prokaryote is *E. coli*.

42. A composition comprising a modified M2 polypeptide of any one of claims 1 to 6 and a pharmaceutically acceptable carrier.

43. A composition comprising a modified M2 polypeptide of claim 7 and a pharmaceutically acceptable carrier.

44. A composition comprising a modified M2 polypeptide of claim 8 and a pharmaceutically acceptable carrier.

45. A composition comprising a modified M2 polypeptide of any one of claims 9 to 11 and a pharmaceutically acceptable carrier.

46. A composition comprising a modified M2 polypeptide of claim 12 and a pharmaceutically acceptable carrier.

47. An antibody to a modified M2 polypeptide of any one of claims 1 to 6.

48. An antibody to a modified M2 polypeptide of claim 7.

49. An antibody to a modified M2 polypeptide of claim 8.

50. An antibody to a modified M2 polypeptide of any one of claims 9 to 11.

51. An antibody to a modified M2 polypeptide of claim 12.

52. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 47.

53. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 48.

54. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 49.

55. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 50.

56. A method of preventing or treating a subject suffering from viral influenza A infection, the method comprising administering a prophylactic or viral load-reducing amount of an antibody according to claim 51.

57. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to any one of claims 1 to 6 and detecting the binding of antibodies to the modified M2 protein.

58. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to claim 7 and detecting the binding of antibodies to the modified M2 protein.

59. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to claim 8 and detecting the binding of antibodies to the modified M2 protein.

60. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to any one of claims 9 to 11 and detecting the binding of antibodies to the modified M2 protein.

61. A method for determining current or previous exposure of a subject to influenza virus, the method comprising contacting a sample from the subject with a modified M2 protein according to claim 12 and detecting the binding of antibodies to the modified M2 protein.

62. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 42.

63. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 43.

64. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 44.

65. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 45.

66. A method of preparing an M2 antibody, the method comprising immunization of a subject with a composition according to claim 46.

ABSTRACT OF THE DISCLOSURE

The present invention provides a method of increasing the recombinant expression and solubility of influenza A virus M2 polypeptide comprising nucleic acids encoding a modified M2 protein of influenza A virus in which transmembrane and other hydrophobic domains have been deleted. The present invention also provides purified polypeptides encoded by the nucleic acids, which polypeptides are immunogenic and are less hydrophobic than full-length M2. Also provided are vaccines comprising variants of M2 expressed in prokaryotic hosts. Further provided are methods of preventing influenza A infection using vaccines comprised of variants of M2. Also provided are antibodies raised against the variants of M2, and use of such antibodies in diagnosis and treatment of influenza A infections.

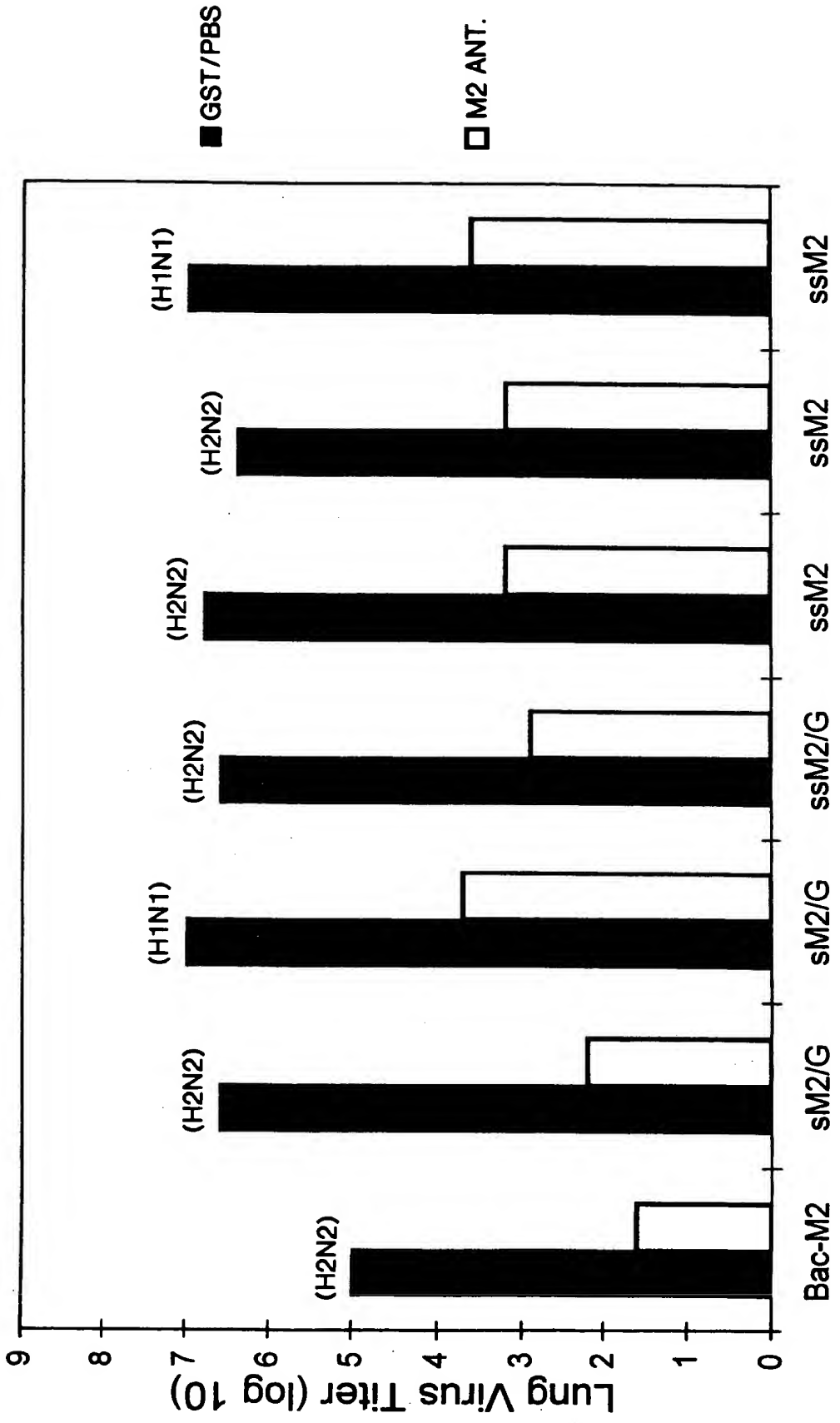
GST/PBS
M2 ANT.
Lung Virus Titer (log 10)
0
1
2
3
4
5
6
7
8
9
(H2N2)
(H1N1)
(H2N2)
(H2N2)
(H1N1)
(H2N2)
Bac-M2
sM2/G
sM2/G
ssM2/G
ssM2
ssM2
ssM2
Antigens

FIG. 4

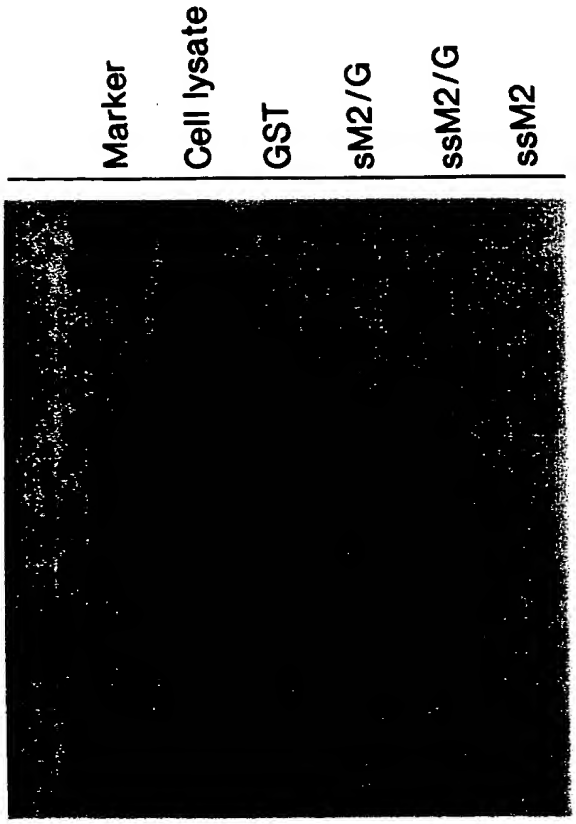

FIG. 3A

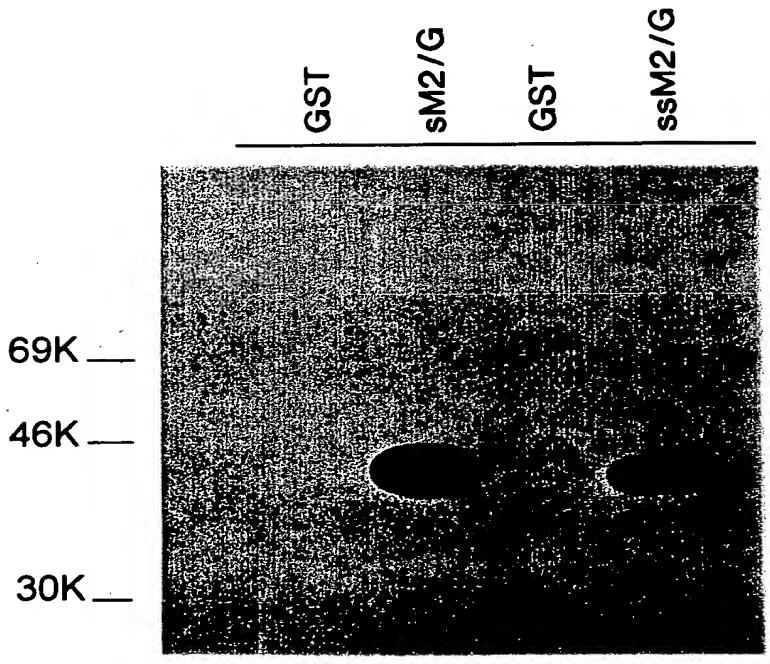

FIG. 3B

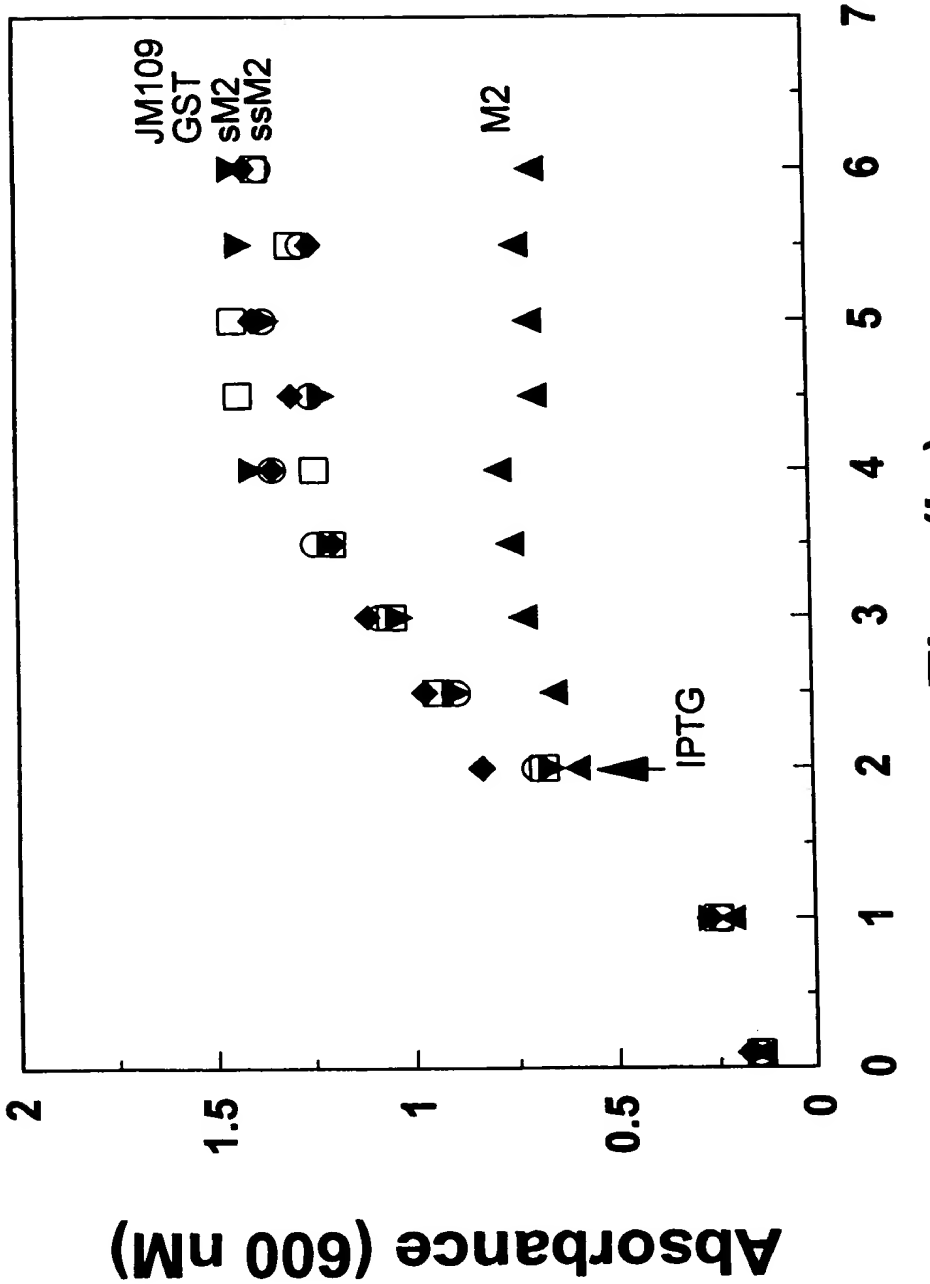
JM109
GST
sM2
ssM2
M2
IPTG
Absorbance (600 nM)
Time (hr)
0
0.5
1
1.5
2
0
1
2
3
4
5
6
7

FIG. 2

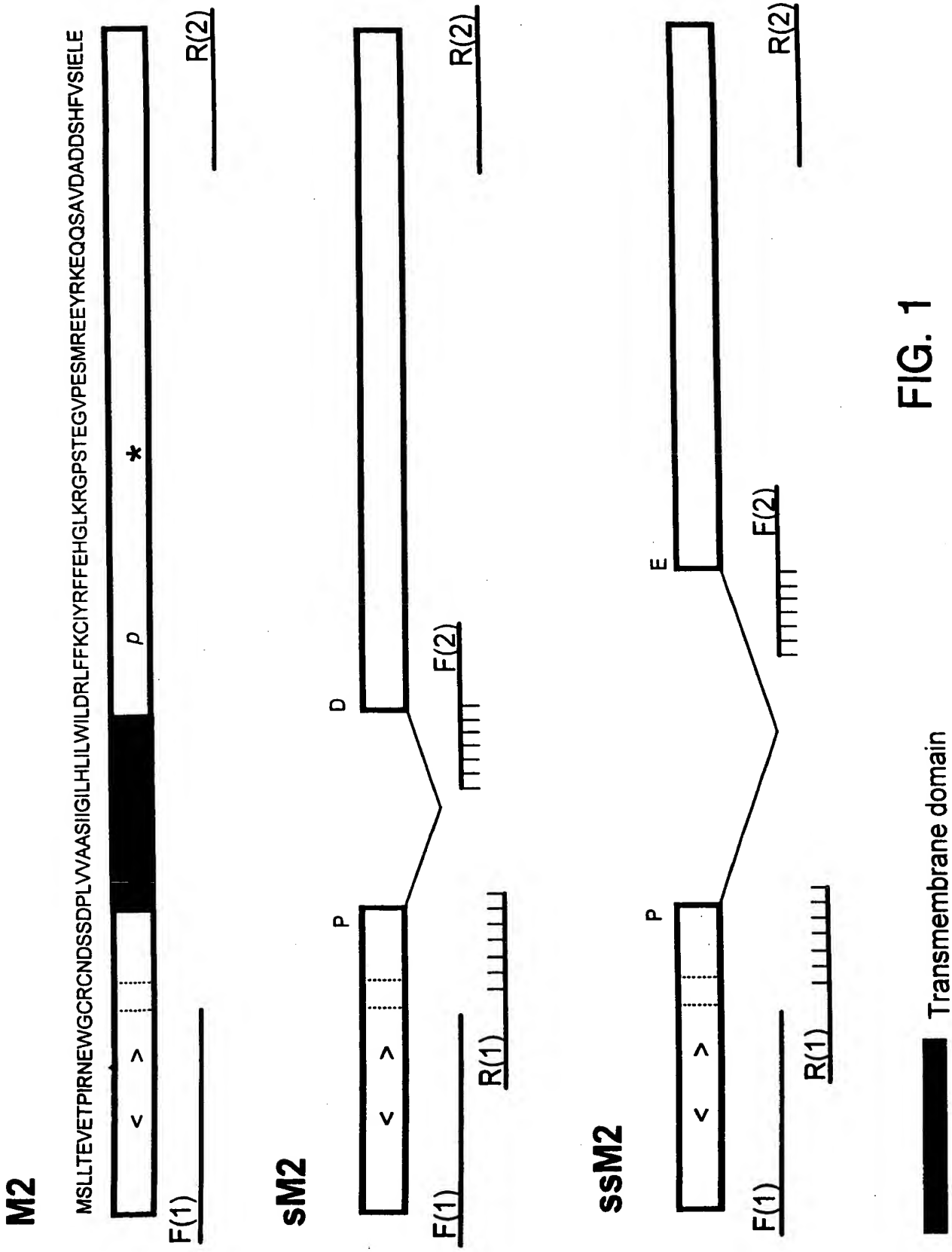
M2
MSLLTEVETPIRNEWGCRCNDSSDPLVVAASIIGILHLILWILDRLFFKCIYRFFEHGLKRGPSTEGVPESMREEYRKEQQSAVDADDSHFVSIELE
p
*
R(2)
F(1)
sM2
D
P
F(2)
R(2)
R(1)
F(1)
ssM2
E
P
F(2)
R(2)
R(1)
F(1)
Transmembrane domain

FIG. 1

SEQUENCE LISTING

<110> Frace, Michael
Klimov, Alexander
Katz, Jaquelline
Centers for Disease Control and Prevention

<120> PREPARATION AND USE OF RECOMBINANANT INFLUENZA A VIRUS M2 CONSTRUCTS AND VACCINES

<130> Modified M2 Protein

<140>
<141>

<150> U.S. 08/906,930
<151> 1997-08-06

<160> 5

<170> PatentIn Ver. 2.0

<210> 1
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Forward-1 primer specific for the 5' coding region of the M2 gene

<400> 1
cccgaattct tatgagcctt ctaaccgagg tcgaaacgcc tatcagaaac gaatggggat 60
gc 62

<210> 2
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: reverse-1 primer coding for 3' amplification from nucleotide 75 of M2 protein.

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<210> 3
<211> 41
<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: Forward-2 primer for sM2 containing a 5' region homologous to the reverse-1 primer

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<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: reverse-2 primer coding for the 3' end of M2.

<400> 4
ctatcagtaa agcagtcgta tctcgacctc atcagctgcc c 41

<210> 5
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Forward-2 primer for ssM2 containing a 5' region homologous to the reverse-1 primer

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caagtgatcc tggaggagga aaacacggtc tgaaaagagg gcc 43